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14. ABSTRACT

Protein phosphorylation plays a key role in the regulation of the function of the proteins and the control of wild range of cellular process. Odin, one of signaling molecules identified in EGF receptor signaling pathway, functions as a negative regulator of growth factor receptor signaling pathways. To dissect the molecular mechanism of Odin in signaling pathway and its biological function, we carried out in vitro kinase assay based on peptide array and identified two new tyrosine phosphorylation site of Odin by c-Src. We also developed two cell lines to study the protein complex of Odin by mass spectrometry. In the future, we seek to identify the domain of Grb2 binding to Odin and the interacting protein(s) of Odin and its phosphorylation site. We also want to characterize the role of the phosphorylation in controlling the function of Odin.

15. SUBJECT TERMS
Odin, Peptide array, phosphorylation, mass spectrometry

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Introduction

Tyrosine kinase mediated signaling events are important for controlling a diverse range of cellular processes ranging from proliferation and cell migration to apoptosis. Dysregulated tyrosine kinase signaling is responsible for a large number of cancers, and several tyrosine kinases are being actively pursued as therapeutic targets for their treatment. Using a mass spectrometry-based proteomic approach, we have previously identified a number of signaling molecules in the EGF receptor (EGFR) signaling pathway. One of the novel molecules, designated Odin, acts as an inhibitor of growth factor receptor signaling pathways. The objective of our work is to investigate the molecular basis of its inhibitory activity, and to establish its role in transformation induced by tyrosine kinases and in mammary development and tumorigenesis.

Body

Since the start of this award, the following progress has been made to characterize the role of Odin in growth factor receptor signaling.

1. Although we have previously shown that Odin undergoes tyrosine phosphorylation in a growth-factor dependent manner, the precise sites of Odin and its upstream kinases are still unknown. Here we developed a peptide microarray-based *in vitro* kinase assay to idenify the candidate phosphorylateion sites of Odin by c-Src (Figure 1). 17 tyrosine-containing peptides derived from Odin (Table1) along with several tyrosine containing peptides derived from a number of other signaling molecules were spotted on glass slides as duplicate (Pepscan Systems, Lelystad, Netherlands) as described earlier (*1*). These custom peptide microarrays were incubated with 50 ng of recombinant c-Src kinase (Invitrogen, Carlsbad, CA) and 200 µM of ^{33}P-labeled ATP (300 µCi/ml , AH9968; GE Healthcare Bio-sciences Corp., Piscataway, NJ) at 25°C for 1 hour in a 120 µl of kinase reaction buffer. After the incubation, the microarray was washed twice in 2M sodium chloride containing 1% Triton X-100, three times in phosphate buffered saline containing Triton X-100 and once in distilled water. The microarrary were then air dried and exposed to the phosphorimager screen for 12 hours and scanned using Biorad Molecular Imager FX (Bio-Rad Laboratories, Inc, Hercules, CA). The image was processed and the intensity values for each spot were obtained using GenePix Pro 6.0 software (Molecular Devices Corporation, Sunnyvale, CA). The relative intensity for each peptide were the average of duplicate spots. In this experiment, one peptide from the linker region of Odin, EEEGP**Y**EALYN (Tyr 361), was highly phosphorylated by c-Src while several other peptides were also moderately phosphorylated by c-Src (Figure 1B and Table 1), which implicate Odin to be downstream of c-Src in receptor tyrosine kinase signaling. Also, the phosphorylation on two tyrosine resides, Tyr 361 and Tyr 455, were also found by us using mass spectrometry (data not shown). Further, global phosphorylation profiling experiments reported by Gygi and his colleagues (*2, 3*) have provided evidence for the *in vivo* phosphorylation of two serine residues in Odin, ATMGSRSE**S**LSNCS (Ser 647) and KKRLEKSP**S**FASEWDE (Ser 663) – both of these sites are also located in the linker region of Odin. These data are exciting because they allow us to perform a systematic mutagenesis study to study the role of these experimentally determined sites in c-Src and in growth factor receptor signaling. They also indicate the importance of the linker region of Odin in a fashion that was not envisioned in the initial proposal.

2. We have generated mouse embryo fibroblasts derived from wild-type and Odin deficient mice (*4*). These cells have been adapted to heavy isotope containing media which will allow us to do an affinity chromatography experiment using anti-Odin antibody generated previously by our group (*4, 5*).

3. In order to find the putative Grb2-binding sites on Odin, we have generated several constructs containing mutations in the putative Grb2-binding sites on Odin (Figure 2). All these mutant are tagged with FLAG, and the expression of these mutants in 293T cells are being examined. Briefly, all these FLAG-tagged mutants will be expressed in 293T cells by transient transfection using Lipofectamine 2000 under the manual (Invitrogen, Carlsbad, CA). 24 hours after transfection, cells will be lyzed and cell lysate will be resolved by SDS-PAGE. The expression of these mutants will be examined by western blotting with anti-FLAG antibodies (Sigma).

Key Research Accomplishments:

In vitro kinase assay has indicated that c-Src can directly phosphorylate Odin.

Future Plans:

1. Our major goal over the next year is to identify the proteins that interact with Odin using the proteomics methodologies that we have optimized over the past year. Once we identify the Odin associated protein complex, we will verify the interactions by systematic in vitro and in vivo experiments using GST and epitope-tagged constructs to determine whether the binding is direct and the exact regions of interaction.

2. We will also use biotinylated peptides derived from the phosphorylated tyrosine residues of Odin to identify the proteins that specifically associate with these sites. The identification of specific binding partners will allow us to better interpret the data obtained from point mutations of these phosphorylation sites.

3. We are excited by the power of the peptide microarrays to reveal enzyme-substrate relationshiops. We will confirm the in vitro observation regarding Odin being a c-Src tyrosine kinase substrate by using chemical inhibitors of c-Src, activators of c-Src, mutant cell lines and phosphospecific antibodies that we plan to generate against the phosphorylation sites that have been discovered by us and by Steve Gygi's group. The phosphospecific antibodies will also allow us to monitor the activation status of Odin easily, especially in tissue sections.

Reportable Outcomes: None.

Conclusion: None

References

1. S. H. Diks *et al.*, *J Biol Chem* **279**, 49206 (Nov 19, 2004).
2. S. A. Beausoleil *et al.*, *Proc Natl Acad Sci U S A* **101**, 12130 (Aug 17, 2004).
3. B. A. Ballif, J. Villen, S. A. Beausoleil, D. Schwartz, S. P. Gygi, *Mol Cell Proteomics* **3**, 1093 (Nov, 2004).
4. T. Z. Kristiansen, M. M. Nielsen, B. Blagoev, A. Pandey, M. Mann, *DNA Res* **11**, 285 (Aug 31, 2004).
5. A. Pandey *et al.*, *Oncogene* **21**, 8029 (Nov 14, 2002).

Appendices: None

Supportting Data:

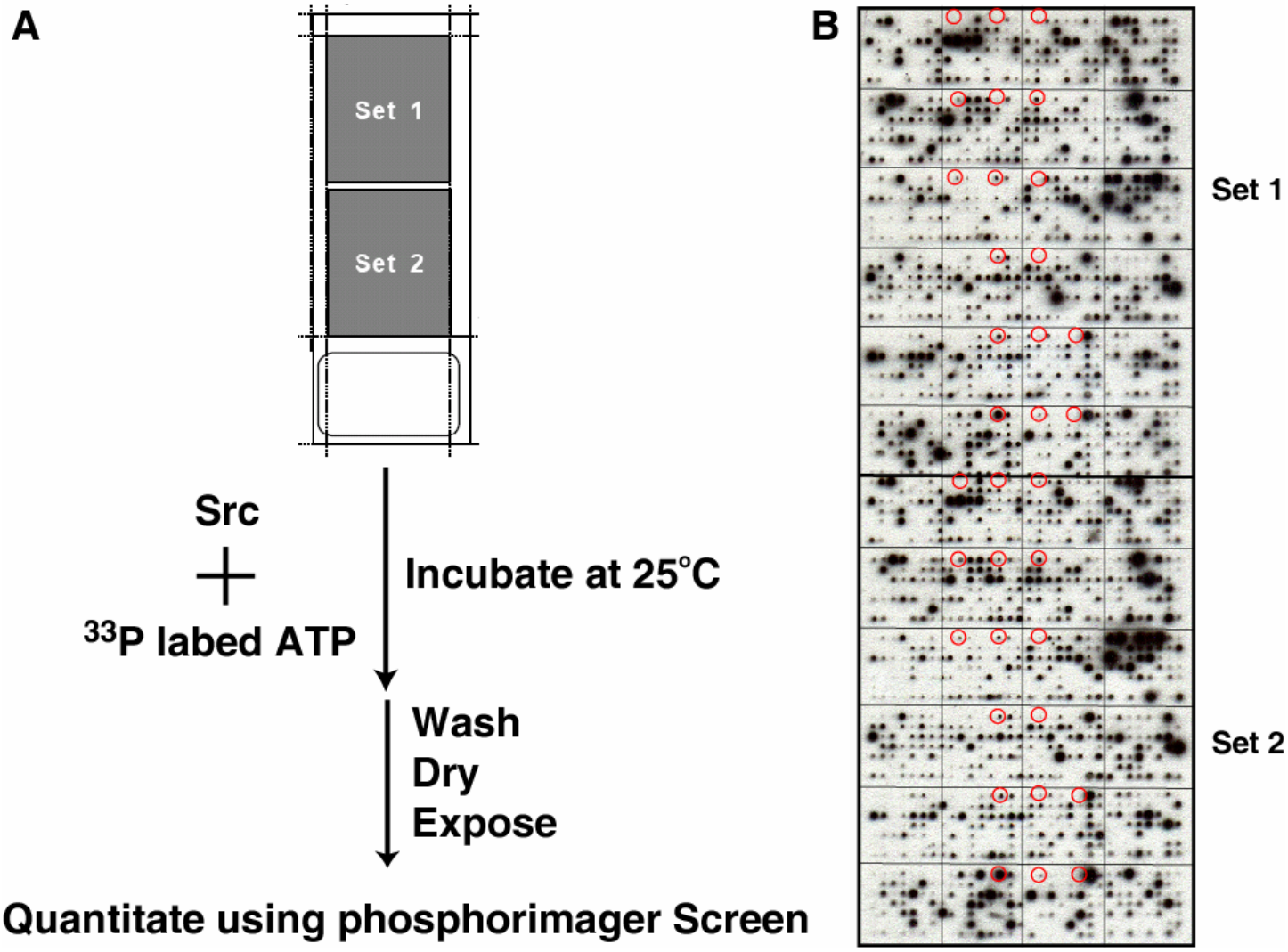

Figure 1. Peptide microarray-based in vitro kinase assay. **(A)**. The peptide microarray was incubated with recombinant c-Src kinase and ^{33}P-labeled ATP at 25°C for 1 hour in a kinase reaction buffer. After the incubation, the microarray was washed, air dried and exposed to the phosphorimager screen. The intensity for each spot was quantitated using phosporimager screen. **(B)**. The autoradiography of the peptide microarray after the *in vitro* kinase assay (see text for details). The glass slide have two sets of peptide microarrays, spotted with the same peptides of the same localization. Each set has 4 × 6 subarrays, and each subarray has 8 × 8 spots. Red circled were spotted with peptides from Odin. Peptide sequence information is listed in Table 1.

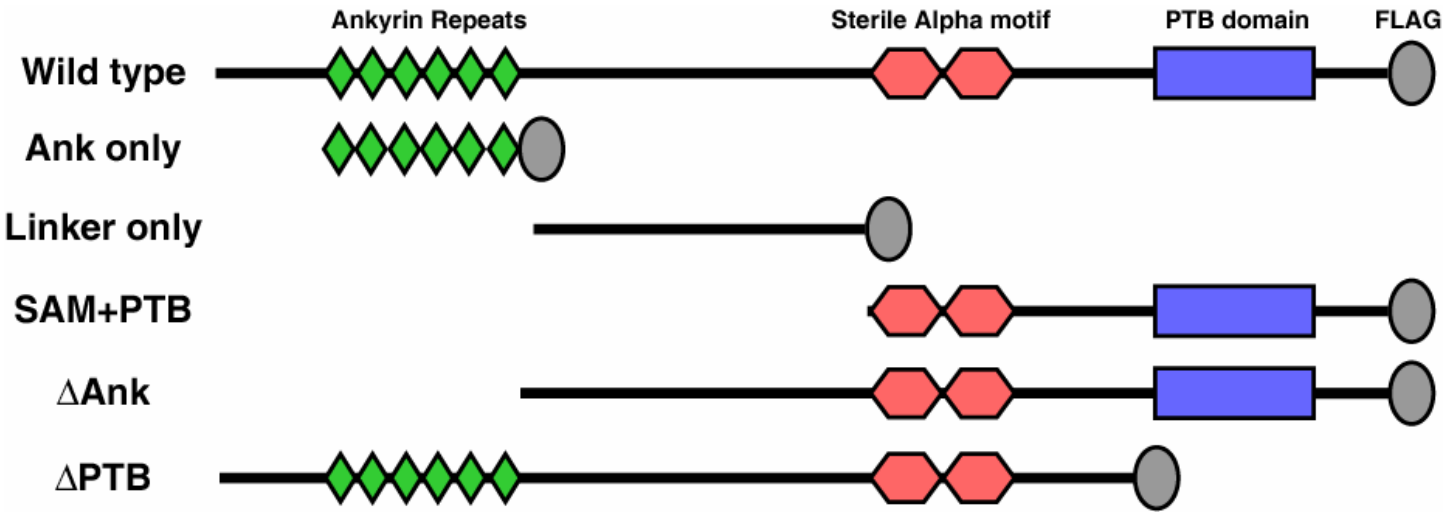

Figure 2. Odin composes of three domains/motifs: six ankyrin repeats motifs (Ank), two sterile alpha motifs (SAM) and one PTB doamin. FLAG-tagged Odin and several FLAG-tagged deletion mutants of Odin have been constructed: ankyrin repeats only, linker region only, SAM and PTB only, Ank deletion (ΔAnk), PTB deletion (ΔPTB).

Table 1. List of 17 tyrosine containing peptides from Odin spotted on the peptide microarray.

X	Y	x	y	Peptide	Relative Intensity
2	1	2	1	GMDSN**Y**QTEMG	9.60
2	1	6	1	VKALG**Y**DGNSP	9.50
2	2	2	1	DLAAL**Y**GRLEV	12.53
2	2	6	1	IGLQQ**Y**ESKLL	10.39
2	3	2	1	VDSTG**Y**TPLHH	10.98
2	3	6	1	SKRVG**Y**LTGLP	13.49
2	4	6	1	EDEHP**Y**ELLLT	12.66
2	5	6	1	HIDKK**Y**FPLTA	14.79
2	6	6	1	EEEGP**Y**EALYN	169.64
3	1	2	1	ILSIT**Y**KGVKF	10.43
3	2	2	1	GYEAN**Y**LGSML	19.96
3	3	2	1	SLAAP**Y**APVQS	9.29
3	4	2	1	LADRP**Y**EEPPQ	9.24
3	5	2	1	FLSSG**Y**SSIDT	9.42
3	5	6	1	AFEVA**Y**QLALQ	9.78
3	6	2	1	LGLQD**Y**VHSFL	9.95
3	6	6	1	DVNLT**Y**EIILT	12.16

X: the column number of the subarray; **Y**: the row number of the subarray; **x**:the column number of the spot; **y**: the row number of the spot.